Role of a-kinase anchoring proteins (AKAPs) in reproduction

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Role of a-kinase anchoring proteins (AKAPs) in reproduction

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1. ABSTRACT

Rapid spatio-temporal organized intracellular signaling is a pivotal mechanism for regulation of functions in many cells, in particular in the female and male gametes, in which functional regulation through rapid increases in protein content is not possible since the mechanisms of transcription/translation are somehow frozen due to meiosis block or DNA condensationrespectively. A kinase-anchoring proteins (AKAPs) represent a functional conserved family of signal-organizing scaffolding proteins, which due to the specific subcellular distribution and focally compartmentalized cyclic-AMP-dependent protein kinase (PKA) and other enzymes, assuring the coordination of cAMP-responsive events and their integration with other intracellular signals. This review summarizes the actual knowledge on AKAP structure and functions, taking into particular account the role of different AKAPs in regulating reproductive functions such as gametogenesis. Evidence for sperm specific AKAP isoforms and their initiated signaling cascades in mature sperm and the role of this focally activated super-molecular signaling complex in motility are discussed in details with particular emphasis on putative relations between AKAP structural and functional alterations and defects in sperm motility.

2. INTRODUCTORY REMARKS

Among the main post-translational mechanisms of regulation of intracellular signaling, the dynamic equilibrium between phosphorylation and dephosphorylation of receptors and intracellular second messengers resultin the activation/blockage of specific signaling pathways, which play a pivotal role. In particular, this sophisticated regulatory mechanism is essential in the female and male gamete, in which functional regulation through rapid increases in protein content is not possible since the mechanism of transcription/translational are somehow frozen due to meiosis block or to DNA condensation respectively. Thus, tuned molecular mechanisms exist in order to strictly and functionally coordinate the spatial and temporal organization of protein kinase and phosphatase systems to generate a controlled and integrated phosphorylation signaling. Scaffold and anchoring proteins dynamically interact with different partners, including kinases and phosphatases, sequestering them in specific cell compartment and allowing the formation of super-molecular protein complexes in which the enzymes can interact with each other, finally leading to a spatio-temporal confined output signal.

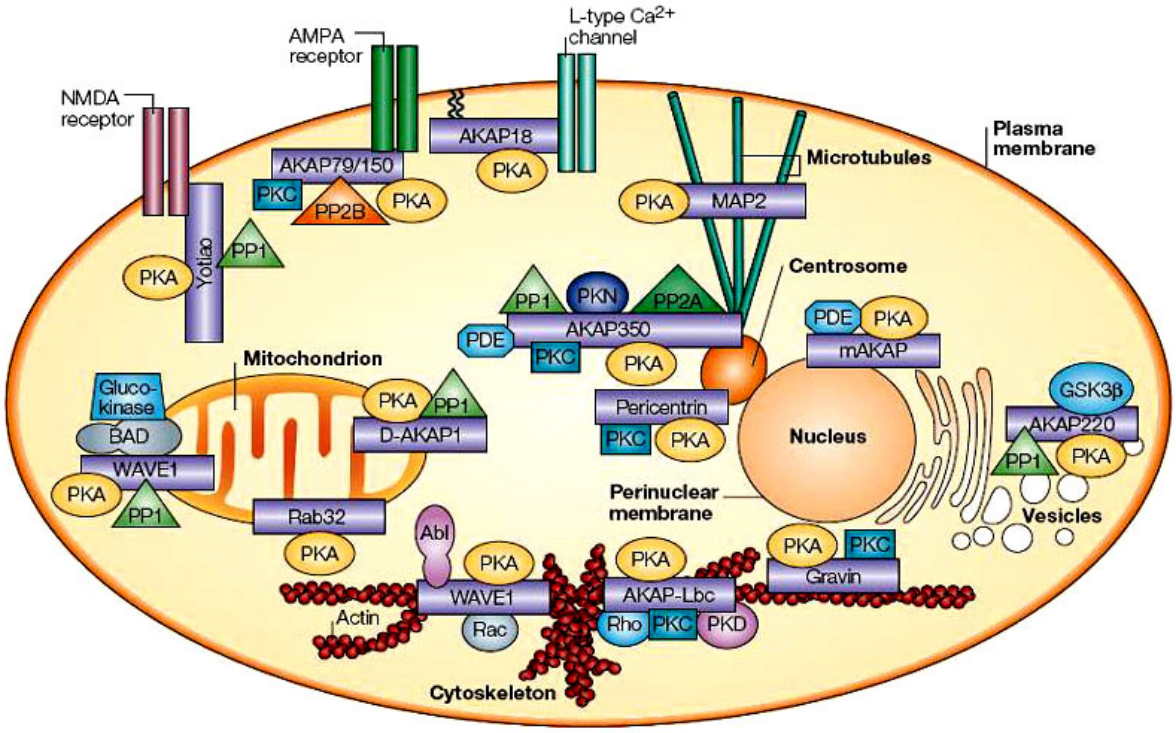

Figure 1. AKAP signaling complexes create compartmentalized focal points for signal transduction. Different A-kinase anchoring proteins (AKAPs) target protein kinase A (PKA) to different cell compartments, including the plasma membrane, the nucleus, mitochondria, cytoskeleton and centrosome. Within a compartment, the same AKAP can associate with different substrates. Alternatively, different AKAPs within the same compartment can assemble distinct signaling complexes. In addition to PKA, AKAPs can interact with other signaling molecules. In particular, with phosphatases (PPTs), and phosphodiesterases (PDEs), which switch off the PKA activated signaling by degrading cAMP. AKAPs also interact with kinases and enzymes from other signaling pathways, such as Abelson kinase (Abl), BCL2-antagonist of cell death (BAD), glycogen synthase kinase-3β (GSK3β), and protein kinases C (PKC) and N (PKN).

One of the wider and functionally most conserved family of these signal-organizing scaffolding proteins is represented by A kinase-anchoring proteins (AKAPs). AKAPs compartmentalize different enzymes and mainly cyclic-AMP-dependent protein kinase (PKA), allowing them to interact and integrate with other intracellular signals.

From the identification of the first AKAP, microtubule-associated protein-2 (MAP2) co-purified with PKA RII regulatory subunit from brain extracts (1), more than 50 AKAPs have been characterized to date (2). AKAPs regulate the precision of the switch on/off of intracellular signaling by coordinating interactions between anchored enzymatic pool of proteins and their substrates at a specific subcellular localization (Fig.1).

AKAP nomenclature is rather confusing and continuously evolving. Originally, AKAPs were classified on the basis of their apparent molecular weight determined by SDS-PAGE or by open reading frame prediction. However, this classification has become cumbersome as some AKAPs originate from an alternative splicing of the same gene and have to be renamed. Moreover, some already described proteins, such as Gravin and Ezrin, have been lately identified as AKAPs, but retain their original general names not related to AKAP family. Finally, some AKAP subfamilies comprise structurally similar orthologues variants expressed in different species with different molecular weight, such as the AKAP79/150, which includes the human AKAP79, the murine AKAP150 and the bovine AKAP75 (2). Integrating sequencing and annotation information, new nucleotide and genome databases number AKAPs sequentially. Tab.1 reports an integrated database in which AKAP new and old nomenclature is reported.

3. cAMP/PKA SIGNALING

cAMP is a diffusible intracellular second messenger produced by adenylyl cyclase (AC) enzymes and destroyed by phosphodiesterases which release the linear AMP molecule. Two families of AC exist: the 9 isoforms of G protein-regulated transmembrane AC (tmAC), responsible of the membrane hormone receptor-initiated-cAMP signaling, and the soluble isoform (SACY) regulated by bicarbonate and intracellular calcium levels, not associated to membrane G-protein coupled receptor (GPCR) and insensitive to forskolin or activated $G\alpha_s$ (Figure 2).

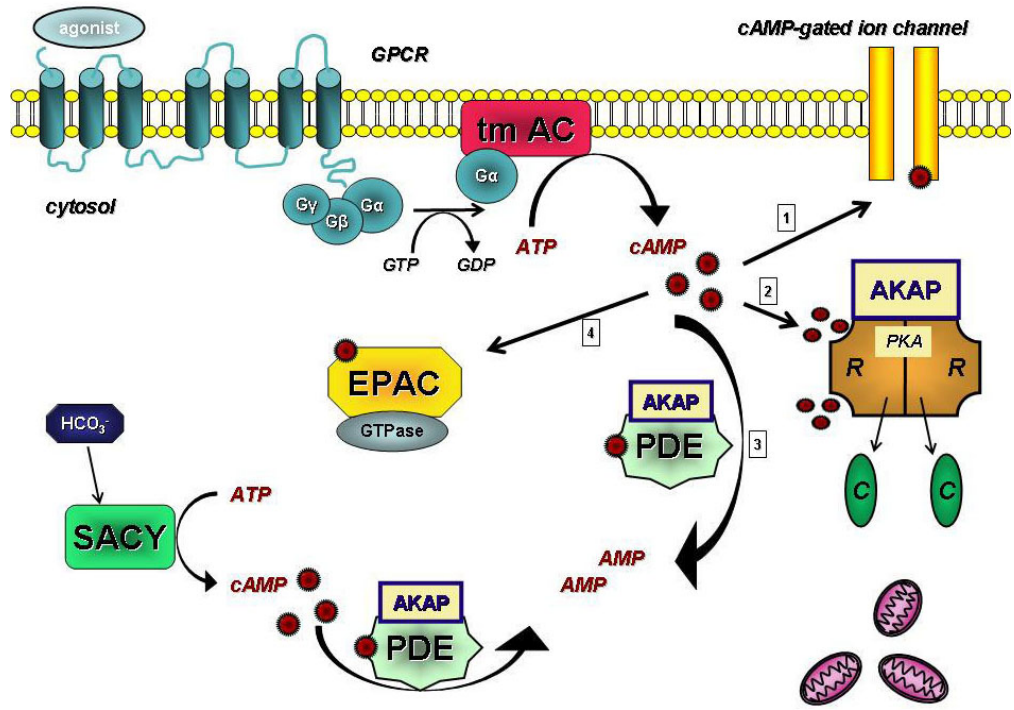

Figure 2. AKAP and cyclic AMP signaling pathways. In the cell cAMP can be synthesized from ATP by to different adenylyl cyclase isoforms: the transmembrane (tmAC), activated by agonist stimulation of G protein-coupled receptors (GPCRs), and in the cytoplasm by the soluble AC (SACY), not associated to GPCR. cAMP can activate nucleotide-gated ion channels (1) or bind the regulatory (R) subunits of protein kinase A (PKA) inducing the release of the active catalytic (C) subunit (2). Moreover, cAMP can trigger phosphodiesterases (PDEs, 3) which catalyze the synthesis of AMP from cAMP, with a feedback mechanism. cAMP activates guanine nucleotide-exchange factors (GEFs) that are known as exchange proteins activated by cAMP (EPACs, 4).

Focal regulation of PKA signaling may be achieved not only by sequestering PKA in specific compartments through interaction with AKAPs, but also by compartimentalized production of cAMP through AC subcellular distribution. Gradients of cAMP accumulating at specific sites within the cells rather than the absolute cAMP intracellular concentrations, regulate activity of cAMP-sensitive proteins, such as cyclic-nucleotide-gated-channels, phosphodiesterases, guanidine nucleotide-exchange proteins activated by cAMP (EPAC). However, the main intracellular target for cAMP is PKA (3). PKA is a tetrameric enzyme consisting of two catalytic subunits (C), which are maintained in an inactive state by binding to a regulatory (R) subunit homodimer. Thus, two PKA subtypes exist depending on the RI or RII regulatory subunits forming the regulatory homodimeric component (Figure 2). Upon binding of cAMP to the regulatory subunits of type I- or type II-holoenzyme, the catalytic subunits are released as active serine-threonine kinases and can actively phosphorylate their specific substrates, initiating a cascade of signaling events inside the cell.

4. STRUCTURE AND FUNCTIONS OF AKAPs

Although they differ for specific sequences which allow interaction with different targets and different sub-cellular compartmentalization, AKAPs share pivotal common properties of scaffolding proteins (Figure 3):

1. a PKA-binding domain
2. a unique localization sequence
3. the ability to form super-molecular complexes with other signaling proteins

4.1.PKA-binding domain

An amphipathic helix of about 14-18 residues which binds the N-terminal dimerization sequence of PKA regulatory subunits is present in all the AKAPs (4). Although the majority of AKAPs specifically bind RII (5), RI-specific AKAPs have also been identified (6). Moreover, very few AKAPs, such as D-AKAP1 (7) and D-AKAP2 (8), show dual affinity properties to bind both PKA regulatory subunits. Following the discovery of the Ht31 fragment of AKAP-Lbc, which has been initially used as an intracellular disruptor of RII-AKAP interaction (9), bioinformatic design of antagonist peptides specific for RII- (10) and RI- (11) AKAP-anchoring sequences has allowed us to discriminate between AKAP initiated signalings mediated by PKA type II and I complex.

4.2.Localization sequence

AKAPs can localize to different subcellular compartments such as the plasma membrane, Golgi,

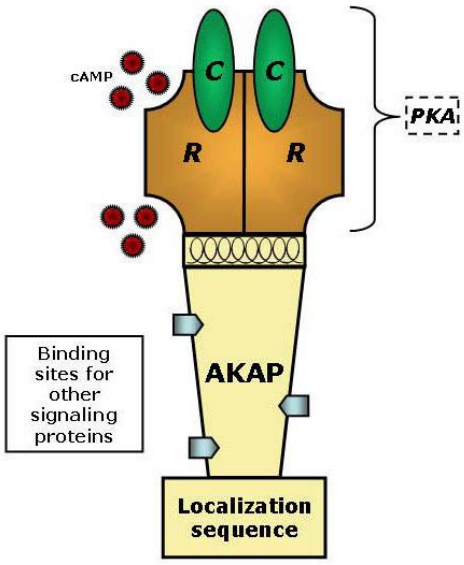

Figure 3. AKAP structure. AKAPs share a similar structure, characterized by an amphipathic helix in the PKA-binding domain, a specific target sequence, and a number of binding sites for other signaling proteins. cAMP; AKAP amphipathic helix in PKA binding domain; Protein kinase A (R= regulatory subunit, C=catalytic one); Binding sites for other signaling proteins.

centrosome, nucleus, mitochondria and cytosol (Figure 1), where they organize focused super-molecular structures, putting together cAMP production, kinases, phosphatases and their substrates (Figure 2). Specific localization is mediated by the presence of specific localization sequences or, as in the case of plasma membrane association, by different docking strategies, including myristoylation and palmitoylation (AKAP18 and gravin), phospholipid binding sequences (AKAP79/150 and Gravin) or unknown mechanisms (Yotiao).

Different AKAPs can be trafficked by different mechanisms to the same subcellular compartments. Subtle differences in the mechanisms of AKAP anchoring could account for their targeting to distinct compartment of the same structure. For instance, plasma membrane anchorage is mediated by phospholipids-recognizing sequence in AKAP79/150 (12) but by myristoylated or palmitoylated groups in AKAP18 (13) and by both phospholipids-recognition sequences and myristoylation in Gravin (14). While D-AKAP1 is targeted for anchorage by a conventional mitochondrial-targeting sequence (15), Wiskott-Aldrich verprolin-homology protein-1 (WAVE-1) is targeted by interaction with BAD (16), or in the case of Rab32, by C-terminal prenylation (17).

On the other hand, alternative splicing gives raise to different AKAP variants from the same gene (Tab. 1), resulting in the association of different targeting sequences to each variant responsible for their different compartmentalization. The long AKAP350 isoform associates with the centrosome by the C-terminal PACT domain (18), while the splice variant Yotiao is targeted to synaptic membranes through binding to the cytoplasmic domain of the NMDA receptor (19).

Besides the existence of a compartment-specific intracellular regulation of composition of AKAP complexes, a more potent mechanism of regulation is represented by the tissue-specific AKAP complex formation. Indeed, signaling complexes are differently assembled on the same AKAP in a tissue specific manner. For instance, depending on the cellular context in which it is expressed, WAVE-1 assembles different PKA partners initiating totally different signalings (2). In the brain, at neurite growth cones, growth factors stimulate actin remodeling and neurite outgrowth through activation of WAVE-1-anchored PKA. Conversely, the same WAVE-1 associates with PKA and different protein targets, such as the metabolic enzyme glucokinase and the pro-apoptotic protein BAD present in the hepatocyte mitochondrial complex, which integrates glycolytic and apoptotic pathways.

4.3.Ability to form super-molecular complexes with other signaling proteins

Scaffolding on specifically compartmentalized AKAPs of all components of the cAMP-PKA system, like AC, phosphodiesterases and PKA, assures the tight spatio-temporal regulation of cAMP signaling through the existence of a specific feedback loop in response to a focused stimulus. In fact, AC-stimulated activity in the focused AKAP complex results in a rapid and localized production of cAMP, activating the anchored PKA with no effect on other compartmentalized PKA, which in turn phosphorylates its anchored substrates initiating the focused signaling cascade (20). Among its substrates, locally stimulated PKA inactivates AKAP-anchored AC by inhibitory phosphorylation as well as activates AKAP-anchored phosphodiesterases and phosphatases by stimulatory phosphorylation dampening the cAMP spike and phosphorylation signaling by a negative and localized feedback loop (20).

In particular, AC anchoring to AKAP provides a sharp mechanism of focused production of cAMP, which results in a spatio- and temporal-regulated activation of PKA associated to the same AKAP. A general AC binding domain has not been characterized in AKAPs, due to the very low homology between AKAP members. In general, different mechanisms assure interaction of the same AC isoforms with different AKAPs and different ACs can interact with different regions of the same AKAP.

Although all the nine tmAC isoforms identified to date can bind AKAP, nothing is known about the ability of bicarbonate-sensitive soluble AC to interact with specific AKAP in order to compartmentalize cAMP production (21). Although a direct interaction has still to be demonstrated, our group has recently shown that AKAP tyrosine phosphorylation associated with increased hyperactivation in human spermatozoa is dependent on cAMP produced by the soluble and not by the tm AC (22)

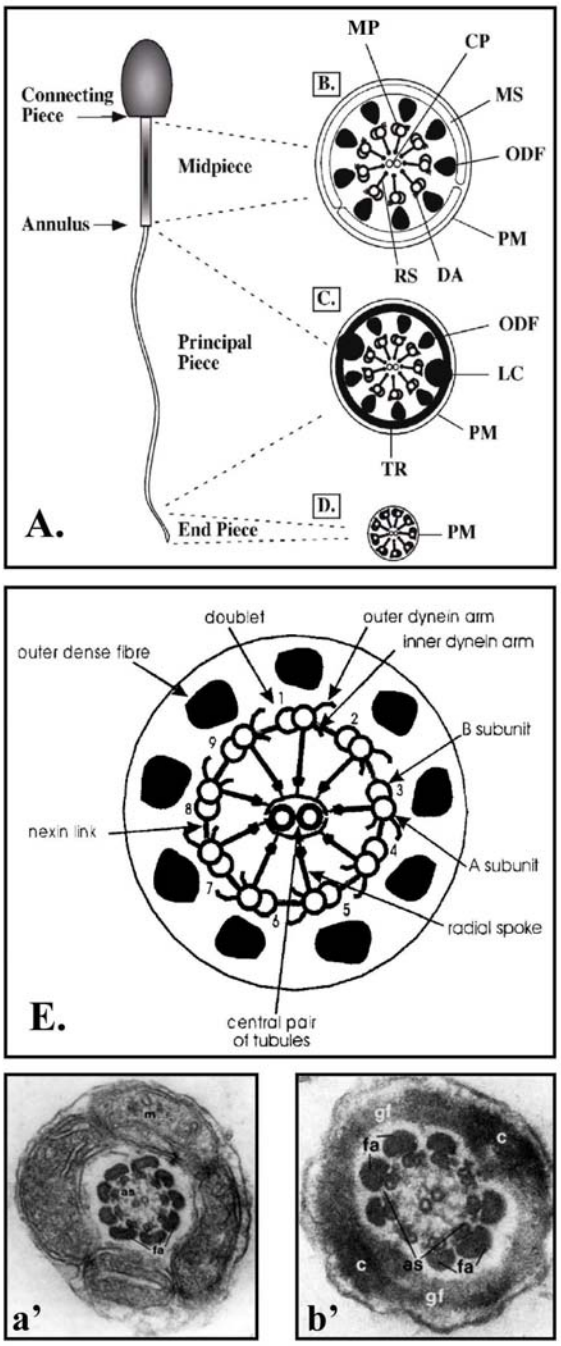

Figure 4. Schematic representation of a human spermatozoa. A. Longitudinal section showing head, midpiece, principal piece and end piece. The insets on the right show the cytoskeletal organization of the sperm tail in transverse sections at different levels: middle (a), principal (b) and end piece (c). Electron microscopy of the transverse sections of the sperm tail at the level of the middle (a') and the principal piece (b') is reported in the lower part of the figure. B. Organization of the axonema: CP: central pair; MS: mitochondria; ODF: outer dense fibers; PM: plasma membrane; DA: dynein arms; RS: radial spoke; MP: microtubule pairs; FS: fibrous sheath. (*From 42, with permission)*

Finally, AKAP orchestration of focused signaling is not a static process, due to the fixed AKAP localization. In fact, upon specific stimulation, different AKAPs, such as Gravin and AKAP79/150, have been described to shuttle on and off from the plasma membrane. In hippocampal post-synaptic membrane, AMPA and NMDA receptor signaling equilibrium underlies fast excitatory synaptic transmissions and long-term depression phenomena, respectively. Both receptors interact with cytoskeletal and signaling proteins, such as PKA and protein phosphatase 2B/calcineurin, through binding with AKAP79/150 in orderto regulate GluR1 phosphorylation and other signaling molecules at post synaptic membranes (23). Upon NMDA activation, AKAP79/150 and the bound PKA are redistributed from postsynaptic membranes to the cytoplasm, resulting in a persistent dephosphorylation and inactivation of postsynaptic substrates such as GluR1, concurring to the NMDA receptor-dependent long-term depression phenomenon (23,24).

5. AKAP REGULATION

Regulation of localization and composition of AKAP complexes can be achieved by different molecular mechanisms. Competition between binding partners may affect AKAP trafficking. For instance, actin and PKA RII compete for the same binding domain on WAVE-1 affecting the cytoskeletal compartmentalization of the complex (25).

Post-translational modifications such as myristoylation and palmitoylation at N terminalenable AKAPs trafficking to plasma membrane. These modifications are reversible, thus making AKAP localization at plasma membrane a dynamic process.

Finally, AKAP phosphorylation resulting from the equilibrium of bound partners such as PKA and phosphatases, modulates subcellular localization of the complex. Phosphorylation of AKAP79/150 by bound PKC results in the release of the anchoring protein from interactions with phosphatidyl inositols of plasmalemma into the soluble fraction (12).

Protein phosphorylation not only regulates AKAP compartmentalization but even affects the activity of anchored enzymes undergoing phosphorylation-dephosphorylation events. Most importantly, AKAP phosphorylation may affect the anchored complex composition by regulating binding of specific partners. Tyrosine phosphorylation of AKAP3 following bicarbonate stimulation or phosphatidyl inositol 3 kinase inhibition in human sperm, results in an increased binding of PKA and its compartmentalization in the fibrous sheath, finally leading to stimulation of sperm motility (22,26). PKA phosphorylation of mAKAP increases its interaction with PDE4D3, resulting in a negative feed back which dampens the initiated cAMP signaling (27). PDE4D3 phosphorylation level in the mAKAP complex integrates in the mAKAP complex integrates cAMP and mitogenic signaling pathways, as PKA-induced phosphorylation in Ser54 enhances phosphodiesterase activity, which is conversely inhibited by ERK5-mediated phosphorylation in Ser 579 (28).

6. AKAP AND REPRODUCTION

AKAPs play important roles in both female and male reproductive systems, in particular during the gametogenesis process. Some of these AKAPs are common to somatic cells, but others are specifically expressed in the female and male gametes and may represent splice variants of somatic AKAPs. A comprehensive list of AKAPs involved in reproductive processes (Tab.2).

6.1. AKAP role in female reproduction

Different functions of female reproductive system are controlled by AKAP activity. Myometral activity is differentially regulated through AKAP79/150 forming protein complexes during pregnancy. In fact, at early stages of pregnancy, hormonal-activated GPCR signaling stimulates tmAC and the sustained levels of cAMP generated at the plasma membrane near the AKAP79/150 complex activates PKA-mediated phosphorylation and inhibition of PLCβ3, leading to uterus relaxation (29). In late pregnancy, PP2B association with AKAP79/150 is accompanied with a concomitant decrease of bound PKA. This triggers a dephosphorylation of PLCβ3, which is capable of being activated by oxytocin stimulation of GPCR, finally leading to an intracellular calcium increase which guides uterine contraction (29).

FSH and estradiol levels affect granulosa cell maturation by lowering AKAP140, which is highly expressed in immature granulosa and increasing synthesis of AKAP80, which drives granulosa cell maturation (30,31). AKAP140 is the ocyte splice variant encoded by AKAP1 gene.

6.2. AKAP role in oogenesis and fertilization

The oocyte meiotic arrest is maintained by PKA activity stimulated by the high levels of cAMP endogenously produced in the oocyte in response to cGMP diffusion from granulosa cells, which inhibits PDE3A (32). LH peak occurring before ovulation determines a rapid closure of the gap junctions between the ooocyte and the somatic cells inhibiting cGMP diffusion. The drop in cGMP removes PDE3A inhibition resulting in a rapid decreased of cAMP and enabling the oocyte to ere-enter meiosis (32). The PKA-mediated inhibition of the maturation-promoting factor (MPF), a complex formed by cyclin B and p34cdc2, whose activation resumes oocyte meiosis, is provided by two mechanisms: a sustained phosphorylation/inhibition of p34cdc2 and the repression of de-novo synthesis of cyclin B1. In response to the preovulatory LH, intraoocyte cAMP concentration drops and MPF is activated by cdc25 phosphatase acting at a yet unknown AKAPx binding complex. The active MPF induces resumption of meiosis by occurring of globular vesicle body formation, chromosome condensation and spindle formation. RII alpha PKA is sequestered to mitochondria by AKAP140, preventing PKA by interfering with the initiated maturation process (33, 34). Interestingly AKAP1 phosphorylation by p34cdc2 kinase of activated MPF, influences the AKAP140/PKA shift from the plasma membrane (35). AKAP140 knock out results in an infertile phenotype in females only, suggesting the pivotal role of this scaffolding protein in oogenesis (36).

WAVE-1 dynamic relocalization within the oocyte is the other major scaffolding player in controlling oocyte maturation and the first phases of fertilization (37). WAVE-1 traffics from the cortex in germinal vesicle oocyte to the cytoplasm in meiosis-II-arrested oocyte. Upon fertilization WAVE-1 concentrates to the male and female pronuclei and redistributes back to the cytosol when nuclear envelope breakdown occurs. Finally, during embryonic cell division it is localized at the cleavage furrow. Altered cellular redistribution of WAVE-1 interferes with a correct process of fertilization, affecting centrosome function and leading to microtubule defects in the aster that would inhibit pronuclear migration. However, the role of such coordinated shuttling and the anchored proteins are still to be fully clarified. Interestingly, association of WAVE1 with PKA is detected in both Met II oocytes and pronucleate zygotes, but interaction with Arp is observed only in Met II oocytes.

6.2. AKAP and the male gamete

Expression of different AKAP isoforms, some of which are uniquely produced in the testis, has been detected in male germ cells during spermatogenesis as well as in mature spermatozoa. Essentially, in the male gamete, AKAPs are involved in both spermatogenesis and activational functions of the mature spermatozoa, such as development and maintenance of sperm motility.

In addition to AKAP3 and AKAP4, which are the main constituents of sperm fibrous sheath and contribute to sperm motility (see below), AKAP220, S-AKAP84 and WAVE-1 expression also appears during spermatogenesis. Although these latter scaffolding proteins have been well characterized and localized in the developing gamete, their precise roles in spermatogenesis are far from being elucidated. In particular, AKAP220 encodes a dual specificity anchoring protein which is expressed throughout spermatogenesis and in spermatozoa. Immunostaining of hAKAP220 dynamically redistributes from a granular cytoplasmic staining in the premeiotic germ cells to a centrosomal localization in the postmeiotic cells and to the midpiece/centrosome area in mature sperm, suggesting two differential stage-specific functions. In fact, the centrosome-associated AKAP220/PKA complex may regulate spindle formation during meiosis and contribute to microtubule organization in the developing flagellum in the postmeiotic phases, where the midpiece-associated complex found in mature spermatozoa may be involved in the development and maintenance of sperm motility (38).

Another member of AKAPs which is spatio-temporal redistributed in the developing germ cell during spermatogenesis is WAVE-1, which localizes with Golgi in the early stages, migrating to mitochondria in the mid-piece in spermatids and mature spermatozoa (39). Following epididymal transit, WAVE-1 is exclusively found on the mitochondrial sheath, suggesting that in haploid cell, WAVE-1 is definitively involved in mitochondrial support to motility. WAVE-1 association with Golgi apparatus in

the early stages may simply reflect the synthesis and sorting of the scaffolding protein, or alternatively, it may be functionally related to a WAVE-1 active role in acrosomal development from Golgi. Indeed, in somatic cells WAVE-1 structurally organizes proteins controlling actin polymerization (40), and the trafficking from Golgi involved in acrosome formation requires a very active actin cytoskeleton.

A sperm specific isoform of AKAP derived from alternative splicing of the product of AKAP1 gene, S-AKAP84, is synthesized as spermatids undergo nuclear condensation and tail elongation (41). The timing of S-AKAP84 expression is correlated with de novo accumulation of RIIα and RIIβ subunits and the migration of mitochondria from cytoplasm (round spermatids) to cytoskeleton (midpiece in elongating spermatids). Immunofluorescence microscopy revealed that the S-AKAP84 is targeted to the mitochondrial sheath during round to elongating spermatid transition (41). It is suggested that PKAII–complexed S-AKAP84 insertion into the outer mitochondrial membrane may drive mitochondrial folding occurring when cells undergo nuclear condensation and tail elongation. During this transition, classically shaped mitochondria dispersed in the cytoplasm of round spermatids migrate to the outer dense fibers of the midpiece cytoskeleton and assume a crescent shape, aligning end-to-end to form the definitive helical array, supporting energy for tail beat (41).

Conversely, a role of the protein in mature spermatozoa is still controversial, since S-AKAP84 is not found in some species in mature spermatozoa, suggesting a species-specific vestigial retention and not an active role in the ejaculated gamete.

6.3.1. AKAP3 beyond sperm

Interestingly, AKAP3 has been recently demonstrated to belong to the so called ‘‘cancer-testis’’ (CT) antigen family. This is a unique class of differentiation antigens highly and specifically expressed in adult male germ cells, but generally not in other normal adult tissues, with the exception of aberrant expression in several cancer types (42). In particular, AKAP3 elevated expression has selectively been found in a great proportion of epithelial ovarian cancers (43,44), but its negative or positive association with the overall and progression-free survival is still controversial.

While the reasons for the aberrant expression of CT antigens in cancer are currently unknown, such a restricted expression in cancer and male gametesmay suggest a link between cancer and spermatogenic processes. In the case of AKAP3, this protein may confer motility properties to cancer cells, contributing to the observed increased metastatic potential and worse prognosis.

6.3.2. AKAP role in sperm motility

In all organisms the sperm mission is to reach the oocyte and transmit the genome to generate a progeny. In order to perform this duty at its best, the spermatozoon generated by spermatogenesis reduces its cell components, such as the as the ribosomes and develops an active propulsory machinery in the flagellum. It also condenses its chromosomal material in order to protect its integrity from external harm which may occur during the long journey to fertilize. Thus, mature spermatozoa are extremely specialized cells unable to perform gene transcription and protein synthesis. In this peculiar context, cell response to stimuli cannot be mediated by new protein synthesis but totally relies on intracellular signaling cascades based on rapid post-translational modifications of second messengers, such as protein phosphorylation/dephosphorylation. In particular, compartmentalization of signaling by scaffolding proteins such as AKAPs becomes a pivotal mechanism to differentiate signaling cascades initiated by different stimuli but propagated by the same bulk of second messengers present in the cell.

Different AKAPs have been identified in spermatozoa from many species, some of which seem to be unique of the testis and of the late stages of spermatogenesis (45). AKAP3 and AKAP4 isoforms are uniquely expressed by spermatids and spermatozoa, predominantly localize in the flagellum and are involved in sperm motility.

6.3.2.1. Structural and molecular bases of sperm motility

The ability to actively swim is gained by spermatozoa during their passage through the epididymis, since testicular spermatozoa are immotile. Spermatozoa display two type of motility which are activated and sustained by different stimuli (46). The forward motility developed in the female reproductive tract allows sperm to detach from seminal plasma in the vagina and actively progress through the uterus, while hyperactivated motility developed under capacitation stimuli in the upper female reproductive tract is essential for the sperm to be able to fertilize. The latter type of motility is characterized by a more vigorous and less symmetric beat of the tail, resulting in a non progressive and whiplash movement, which seems to be important for sperm detachment from the uterus epithelium and for mechanical progression through the cumulus oophorus and zona pellucida surrounding the oocyte. Sperm swimming is characterized by a rhythmic three-dimensional and asymmetric movement of the flagellum. This is a peculiar type of cilium, whose structure get assembled in elongating spermatids and proceeds distally from the cell body during the final stages of spermatogenesis.

In the principal tract, the flagellum is arranged in a cytoskeletal structure organized in the axonema, common to all cilia, which is surrounded by 9 outer dense fibers (ODFs). Two opposite ODFs fuse with two surrounding longitudinal columns, which are connected throughout the tail by semilunar ribs. This structure consisting of ODF, lomìngitudinal columns and seminlunar ribs is called fibrous sheath and is peculiar of sperm flagellum (Fig.4). Conversely, in the middle piece of the flagellum the fibrous sheath is replaced by packed mitochondria, providing part of the energy necessary for tail beating. The reciprocal

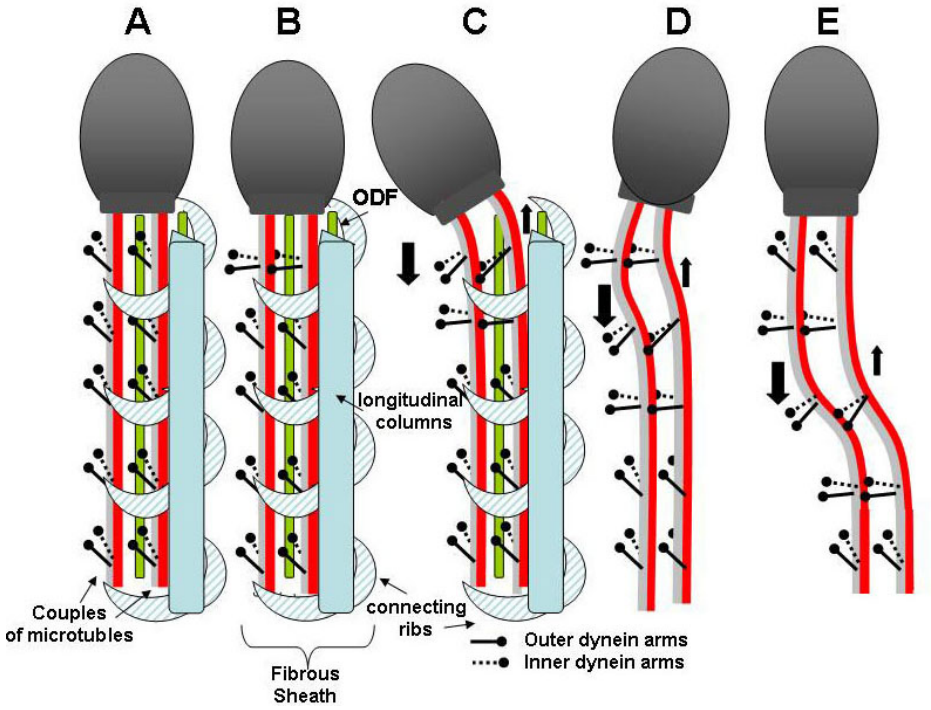

Figure 5. Mechanism of sperm tail movement based on the reciprocal sliding of two adjacent couples of microtubules and on the microtubule anchoring to the fibrous sheath. Longitudinal section of human spermatozoon. Two adjacent couples of microtubules (grey and red), the fibrous sheath with the ODF (green) and longitudinal column as well as semilunar ribs (light blue) are shown. The bending of the flagellum is due to the subsequent cycles of dynein arm attachment, generation of forces and detachment on the B subunit of the adjacent microtubule couple. The anchoring of the microtubules on the fibrous sheath results in deformation of the beat generating rhythmic three-dimensional and asymmetric movement of the flagellum. A-E: sequential reciprocal sliding of 2 adjacent couples of microtubules in the sperm principal piece. To better appreciate the sliding movement, the fibrous sheath with ODFs (green) and longitudinal columns as well as semilunar ribs (light blue) have been removed from panel D and E.

sliding of each couple of microtubules originating from the sequential anchoring of the dynein arms on the neighbor couple and ATP-dependent generation of sliding force finally result in bends of alternating direction and propagation of the oscillation along the tail (Figure 5). The connection of microtubules to the rigid fibrous sheath and the presence of ODF asymmetrically restrain the planar beat, conferring a three dimensional helical shape to the propagating flagellar movement (Figure 5). Energy to support such a process is represented by ATP which is hydrolyzed by dynein ATPase to generate the sliding force in the microtubules. Although the main source for ATP has long been considered the oxidative phosphorylation in the midpiece mitochondria, a local production of energy through an alternative glycolytic pathway has also been characterized in the sperm principal piece, which is mainly involved in sustaining hyperactivated motility (47). Interestingly, the sperm specific glycolytic enzyme isoforms glyceraldehyde 3-phosphate dehydrogenase (GAPDS) and to a lesser extent hexokinase 1 (HK1), have been characterized and found associated with the fibrous sheath (48,49). Thus, the fibrous sheath represents a functional scaffold for integrating glycolityc enzymes and signaling molecules involved in controlling sperm motility. The sliding of microtubules depends on cAMP levels and the velocity of the sliding is ATP-dependent.

6.3.2.2. AKAP3 and AKAP4

The fibrous sheath is mainly composed by two sperm specific AKAP isoforms, AKAP3 and 4, which localize in the connecting ribs or in the longitudinal columns, respectively. Although in the mouse AKAP4 is the main component of the FS (50), in humans AKAP3 seems to be the predominant AKAP present in sperm lysates (51). Both proteins are transcribed and synthesized postmeiotically during spermatogenesis and are localized in the principal piece of the sperm tail. They bind the PKA I and II complexes showing dual specificity for PKA regulatory subunits. Human sperm AKAP3 has been cloned and characterized simultaneously in Carr's (AKAP110, 51) and Herr's laboratories (FSP95, 52). Moreover, Lefevre et al (53) reported cloning SOB1, a new testis specific human sperm protein. Although SOB1 shares 99% sequence identity with FSP95 and maps to the same 12p13.3 locus, it localizes to the sperm outer surface, being involved in oocyte binding. To our knowledge, such peculiar function and localization has never been further confirmed by any laboratories. Although some differences have been found between FSP95 and AKAP110 by the two groups, they represent the same protein sharing 40% homology with AKAP4. Indeed, our group used the two antibodies raised against FSP95 or AKAP110 (kindly provided by Carr's and Herr's laboratories) to detect the same AKAP3 in human

sperm lysates (22,26,54). We also found a faint positivity for AKAP3 in sperm acrosome (46,51), supporting the role of AKAP-PKA as a signaling complex in the progesterone-initiated acrosome reaction (55). Interestingly, it has been suggested that since sperm contain both RI, localized in the acrosomal region, and RII, exclusively localized in the tail, AKAP3 may differentially activate independent signaling cascades in the two compartments by interacting with PKA I complex at the acrosome or with PKA II in the principal piece (51).

AKAP4 (previously named AKAP82, see Tab.1) has been the first AKAP to be described as associated with the sperm flagellum (50). A highly conserved protein of about 82 kDa has been characterized in mouse (50,56), bovine (57) and human (58) spermatozoa. It is synthesized as a precursor of around 97 kDa (pro-AKAP82) in round spermatids.and processed to a the full mature AKAP4 in condensing spermatids. In mature spermatozoa, the precursor is not detectable, suggesting that it could play a role in flagellar development. It has been hypothesized that fibrous sheath proteins synthesized in the spermatid body could be trafficked to the axoneme as a complex with the assembly-incompetent AKAP4 precursor. Once cleaved to the active form, AKAP4 would drive the correct fibrous sheath folding (59).

Both AKAP4 and its precursor are actively phosphorylated in tyrosine during human sperm capacitation, although this effect does not seem to be associated with increased motility. Moreover, since in human spermatozoa there is a less extent of processed AKAP4 compared to other species, which also show higher sperm motility, a correlation between the degree of AKAP processing and motility has been postulated. However, such relation has never been proven, since no difference in relative amounts of pro-AKAP4 and AKAP4 has ever been detected in human sperm samples following capacitation and induction of sperm motility (60). Moreover, the decrease motility observed following cryopreservation and thawing of equine spermatozoa is not associated with a decreased binding of AKAP4 and PKA in the flagellum (61).

AKAP4 maps to the proximal end of the X chromosome (Xp11.2) in the mouse (50) and in the syntenic region in humans (58), thus, defects in the gene are maternally inherited. Moreover, since the gamete is haploid when the transcript of pro-AKAP4 first appears, the AKAP4 mRNA or protein must be redistributed between conjoined spermatids through intercellular bridges in the later stages of spermatogenesis.

6.3.2.3. AKAP genetic defects and asthenozoospermia

Since AKAP3 and 4 are involved in sperm motility, defects in the encoding genes or in the post-translational modifications affecting pro-AKAP and AKAP functions (such as proteolitic cleavage, capacitation-induced phosphorylation) could be present in patients showing genetically-based motility defects. Genetic sperm defects affecting the flagellar structures and resulting in asthenozoospermia include: 1. *stump and short tail* defects, characterized by reduced length of the tail and defects in the flagellar structures; 2. *immotile cilia syndrome*, characterized by absence of the dynein arms; 3. *dysplasia of the fibrous sheath*, including all defects in this tail structure resulting in an abnormal flagellum and severely malformed fibrous sheath; 4. *detached tails*, characterized by headless tails; 5. *9+0 axoneme* and *absence of axoneme*, with the absence of the central microtubule couple or of the entire axoneme. Although in a stump tail case, no AKAP4 was detected (62), this is probably secondary to the main yet unknown defect, since no quantitative and qualitative alteration in AKAP3 and in AKAP4 and its precursor proAKAP4 has been found in dysplasia of the fibrous sheath (63) and stump tail (64) pathologies. In fact, AKAP3 and 4 localize correctly to the fibrous sheath of the amorphous flagellum and retain the ability of interact with each other and with PKA (63) in the overlay assay. However, the ability of such anchoring proteins to undergo functional phosphorylation, one of the post-translation modifications which affects PKA compartmentalization and activation signaling (26), has never been investigated. Moreover, fibrous sheath dysplasia may arise from different gene mutations, as suggested by the presence of partial deletions in the akap3 and akap4 genes and absence of AKAP4 protein in the fibrous sheath in a case report (65). Total absence of AKAP4 has even been reported in a case of necrozoospermia (66).

Targeted disruption of the *akap4* gene by CRE/loxP method resulted in defects in sperm flagellum and motility (67). Knock out mice for AKAP4 produce normal numbers of spermatozoa but fail to develop progressive motility, have shortened flagellum and have reduced or absent proteins which are normally associated and coordinated in function by AKAP4, such as RII alpha, AKAP3, GAPDS. Thus, absence of AKAP4 results in altered flagellar structure and function. Structural defects are very specific. In fact, the axonemal and outer dense fibers components of the cytoskeleton are correctly formed, since their assembly in the developing flagellum is independent of the fibrous sheath. Conversely, the fibrous sheath, of which AKAP4 is the main component is incomplete, resulting in a thinner principal piece and a shorter flagellum with an amorphous structure. This altered structure is no longer able to correctly recruit and orchestrate the signaling underlying sperm motility.

Development of an AKAP3 knock out mouse model would help the study of the differential functions of AKAP3 and AKAP4 in regulation of motility (68) but also in the flagellar assembly. In fact, AKAP3 and AKAP4 are differently located in the fibrous sheath, being AKAP3 confined to connecting ribs only and AKAP4 in both ribs and longitudinal columns. This different localization may be important not only for sperm motility but also for flagellar assembly. Moreover, although their transcription begins in early spermatid development, AKAP3 is synthesized in round spermatids and incorporated into the fibrous sheath concurrently with formation of the rib precursors, while AKAP4 is synthesized and incorporated into the nascent fibrous sheath late in spermatid development (69). The AKAP4 precursor is processed in

Table 1. AKAP nomenclature

Common Name	Official Name (from encoding gene)	Alternative Name and splice variants	Chromosome	Tissue expression	Cell Localization	RefSeq IDs	Swiss Prot Ref	Gene ID
D-AKAP1	AKAP2AKAP1	AKAP84, AKAP140, SAKAP84, PRKA1, AKAP121, AKAP149, MGC1807,	17q21-q23	Testis, ovary, prostate, intestine, kidney, pancreas, liver, lung, colon carcinoma	Mitochondria outer membrane	NM_003488	Q92667	8165
PALM2	AKAP2	AKAP-KL, KIAA0920, DKFZ,p564L0716, FLJ53197	9q31-q33	Cell membrane, lipid-anchor, cytoplasmic side	Heart and muscle, and fibroblasts	NM_001004065	Q9Y2D5	11217
AKAP3	AKAP3	FSP95, SOB1, AKAP110, CT82	12p13.3	Testis, sperm	Fibrous sheat and acrosome	NM_006422	O75969	10566
AKAP4	AKAP4	AKAP82, CT99, FSC1, HI, PRKA4, hAKAP82, p82	Xp11.2	Testis, sperm	Fibrous sheat, cilium, flagellum	NM_003886	Q5JQC9	8852
AKAP79	AKAP5	AKAP75, AKAP150, H21	14q21-q24	Cerebral cortex and postsynaptic densities of the forebrain, adrenal medulla, ovary, lung and anterior pituitary	Plasma membrane	NM_004857	P24588	9495
mAKAP	AKAP6	ADAP100, ADAP6, AKAP100, KIAA0311, MGC165020, PRKA6	14q12	Highly expressed in cardiac and skeletal muscle Brain	Sarcoplasmic reticulum, nucleus membrane	NM_004274	Q13023	9472
AKAP18	AKAP7	AKAP15	6q23	Brain, heart, lung, pancreas and placenta	Cytoplasm	NM_004842	Q9P0M2	9465
AKAP95	AKAP8	DKFZp586B1222	19p13.1	Heart, liver, skeletal muscle, kidney and pancreas	Nucleus matrix	NM_005858	O43823	10270
YOTIAO	AKAP9	KIAA0803, AKAP350, AKAP450, CG-NAP, HYPERION, PRKA9, MU-RMS-40.16A	7q21-q22	Widely expressed. Isoform 4: skeletal muscle and pancreas	Cytoplasm, cytoskeleton, centrosome, Golgi	NM_005751	Q99996	10142
D-AKAP2	AKAP10	PRKA10, MGC9414	17p11.1	Liver, lung spleen, brain	Mitochondria, cytoplasm	NM_007202	O43572	11216
AKAP220	AKAP11	DKFZp781I12161, FLJ11304, KIAA0629, PRKA11	13q14.11	Heart, brain, lung, liver, kidney, testis and ovary. Less in skeletal muscle, pancreas and spleen	Cytoplasm, cytoskeleton, centrosome	NM_016248	Q9UKA4	11215
GRAVIN	AKAP12	AKAP250, DKFZp686M0430, DKFZp686O0331, FLJ20945, FLJ97621	6q24-q25	Endothelial cells, fibroblasts and osteosarcoma	Cytoplasm, cytoskeleton	NM_005100	Q02952	9590
AKAP-Lbc	AKAP13	ARHGEF13, BRX, FLJ11952, FLJ43341, HA-3, Ht31, LBC, PROTO-LB, PROTO-LBC, c-lbc	15q24-q25	Isoform 2 : heart, lung, placenta, kidney, pancreas, skeletal muscle and liver Isoform 3 & 6: hematopoietic cells, skeletal muscle, lung, heart, estrogen-responsive reproductive tissues Isoform 6: not found in brain, placenta, liver, pancreas ,kidney Isoform 7:myeloid & lymphoid lineages, epithelial tissues,	Isoform 2: Cytoplasm Isoform 3: Cytoplasm, nucleus Isoform 6: Cytoplasm Isoform 7: Membrane	NM_007200	Q12802	11214

				skeletal muscle				
AKAP28	AKAP14	-	Xq24	Tissues containing axoneme-based organelles (cilia and/or flagella)	Cytoplasm	NM_178813	Q86UN6	158798
WAVE1	WAVE-1	FLJ31482, KIAA0269, SCAR1, WAVE	6q21-q22	Brain., testis, ovary, colon, kidney, pancreas, thymus, small intestine and peripheral blood	Cytoplasm, cytoskeleton	NM_001024934	Q92558	8936
PERICENTRIN	PCNT	KEN, MOPD2, PCN, PCNT2, PCNTB, PCTN2, SCKL4	21q22.3	Ubiquitus	Cytoplasm, cytoskeleton, centrosome.	NM_006031	O95613	5116
EZRIN	EZR	AKAP78, CVIL, CVL, DKFZp762H157, FLJ26216, MGC1584, VIL2	6q25.2-q26	Cerebral cortex, basal ganglia, hippocampus, hypophysis, and optic nerve. Stronger expression in gray vs white matter. Intestinal epithelial microvilli, astrocytes	Apical and peripheral cell membrane, cytoplasm, cytoskeleton.	NM_003379	P15311	7430
MAP2	MAP2	DKFZp686I2148, MAP2A, MAP2B, MAP2C	2q34-q35	Ubiquitus	Cytoplasm; cytoskeleton	NM_001039538	P11137	4133

According to http://www.genenames.org/genefamily/akap.php modified and integrated with NCBI and SwissProt databases; AKAP common, official and alternative nomenclature as well as the name of splice variants, chromosome locus, tissue and subcellular localization are indicated.

Table 2. AKAPs involved in reproduction

Common Name (splice variants)	Official name	Role	Tissue expression	Cell Localization	References
AKAP140	AKAP1	Cell maturation Meiosis control	Granulosa cells Oocyte	Plasma membrane Mitochondria	30,31 33-36
AKAP79/150	AKAP5	Uterus contraction/ relaxation	Myometrial cells	Plasma membrane	29
AKAP80	-	Cell maturation	Granulosa cells	Plasma membrane	30,31
WAVE-1	WAVE-1	Oocyte maturation Fertilization, first division in the zygote Acrosome development, sperm motility	Oocyte Zygote Male germ cells	Cortex, cytosol Pronuclei, cytosol, cleavage furrow Golgi, mitochondria	37 39,40
AKAP3	AKAP3	Sperm motility, fibrous sheath folding Acrosome reaction	Spermatids, spermatozoa	Fibrous sheath of sperm tail acrosome	22,26,46,51,54,70- 76, 82 46,51,55
AKAP4	AKAP4	Sperm motility, fibrous sheath folding	Spermatids, spermatozoa	Fibrous sheath of sperm tail	50,56-59, 60,61, 70,71
SOB1	AKAP3	Oocyte binding	Spermatozoa	Sperm outer surface	53
SAKAP84	AKAP1	Mitochondria folding	Spermatids, spermatozoa?	Mitochondria	41
AKAP220	AKAP11	Spindle formation, microtubule organization in flagellum, sperm motility	Male germ cells	Cytosol, centrosome, midpiece	38

The common name or the name of the splice variants as well as the official name of the full length protein are indicated together with the function in the cell, tissue expression and cell localization

the flagellum and only the mature form of AKAP4 appears to bind AKAP3. These results suggest that during the flagellar development, AKAP3 is involved in organizing the basic structure of the fibrous sheath, whereas AKAP4 has a major role in completing fibrous sheath assembly.

6.3.2.4. AKAP3 / AKAP4 platform and sperm motility

Besides the role in driving the flagellum assembly during spermatogenesis, both AKAP4 and 3 are essential for development and maintenance of sperm motility. Their coordinated ability is essential to recruit and organize different enzymes into a signaling platform in the fibrous sheath. Several kinases, phosphatases and phosphodiesterases as well as cytoskeletal proteins contribute to the transduction of a correct signaling cascade supporting motility and have been described to coordinately interact and be activated by the AKAP3 and AKAP4 platform. In particular, during capacitation AKAP3 and 4 activity can be regulated by phosphorylation in specific aminoacids. Some of the AKAP3 and AKAP4

phosphorylation sites have been mapped and correspond mainly to tyrosine residues and serine residues (70,71). In particular, one of the phosphorylation sites corresponds to the PKA binding site mapped in a previous paper by the same group (72).

Focused and compartmentalized cAMP production and dampening is selectively regulated by the coordinated interaction of PKA and phosphodiesterases with AKAP3 and AKAP4. The PKA complex is selectively targeted to the fibrous sheath by increased tyrosine phosphorylation of AKAP3 (26). This process physiologically occurs during capacitation (51) and activation of SACY by bicarbonate (54) or by the pharmacological inhibition of PI3 kinase activity (26), resulting in a localized activation of PKA. Moreover, a selective binding of AKAP3 to PDE4A has been demonstrated by pull down experiments in bovine sperm tail (73). Inhibition of PDE4A (73) as well as PKA catalytic alpha subunit (74) results in increased cAMP production and progressive motility, suggesting a regulatory model of cAMP production in which PKA stimulates PDE4 phosphodiesterase activity at the level of AKAP3 complex which dampens cAMP in a spatio-temporal regulated feed back.

Among the several proteins that are able to interact with different AKAPs in addition to PKA regulatory subunits, a family of proteins sharing a conserved RII dimerization/docking (R2D2) domain which binds the amphipathic helix of AKAPs, has recently been characterized in mammalian spermatozoa. These R2D2-containing proteins consist of ROPN1, ASP, SP17 and CABYR (75-77). These proteins cannot bind cAMP and they share no other conserved domain with RII, suggesting a role other than PKA regulation. In particular, although originally thought to be confined to sperm flagella, these four proteins have been demonstrated to be widely expressed in a variety of tissues containing motile flagella (SP17, CABYR and ASP) and primary cilia (SP17). Pull down experiments clearly showed that all R2D2-containing proteins bind not only AKAP3 (74), but also AKAP1, MAP2D and RSP3. They probably exert different functions depending on the specific expression pattern of each AKAP (77). Binding of these proteins to AKAP3, in particular ropporin, a sperm specific rhophilin-binding protein localized in the fibrous sheath (78), is increased by AKAP phosphorylation, similarly to what happens to RII (26,76). Interestingly, the amphipatic Ht31 peptide corresponding to the RII binding domain of AKAP can compete with AKAP bind all the four proteins. These findings suggest that inhibition of sperm motility (26,51) and acrosome reaction (55) observed following S-Ht31 addition may be due not only to disruption of interaction of AKAP with PKA but also with other proteins, such as R2D2-containing proteins. A R2D2 knock out mouse model will be important for further elucidating the role of this motif and of the R2D2-containg proteins in cilia functions and fertility. The infertile AKAP4 knock out mice show normal distribution and activity of all AKAP interacting proteins. Only the cell distribution of PKA catalytic and its regulatory subunits seems to be altered, along with PI3K and SP17 (79). A two fold increase activity of the major sperm serine/threonine phosphatase PP1□2, may be due to a decrease of its inhibitory phosphorylation (80).

Phosphorylation of AKAP3 regulates its ability to bind and activate all the interacting R2D2-containg proteins. In particular, in bovine spermatozoa the components of the RhoA-ROCK-cofilin signaling cascade which modulates actin polymerization involved in membrane remodeling preceding acrosome reaction (81) have been demonstrated to be recruited and activated by AKAP3 phosphorylation (76). According to this model, AKAP4-induced phosphorylation of AKAP3 increases its interaction with RhoA-interaction proteins, such as PKA RII and ropporin, suggesting that ropporin binding of RhoA through rhophilin may finally activate the RhoA-mediated actin polymerization process, contributing to acrosome reaction (76). Calcium signaling does not seem to affect AKAP3 ability to bind either ropporin or the calcium-binding protein CABYR, whose interaction with AKAP3 is not even modulated by phosphorylation (76).

AKAP phosphorylation may initiate different signaling according to AKAP compartmentalization in the spermatozoon. In fact, while AKAP3 phosphorylation and activation in the acrosome contributes to the acrosome reaction through the activation of the RhoA cascade, in the tail, AKAP3 phosphorylation increases AKAP3 recruitment and activation of signaling involved in regulation of sperm motility. Our group has demonstrated that tyrosine phosphorylation of AKAP3 in human spermatozoa results in selective recruitment and activation of PKA in the fibrous sheath through direct interaction of the RII beta regulatory PKA subunit with phosphorylated AKAP3 (26, Fig.6). Inhibition of tyrosine phosphorylation of AKAP3 or RII beta binding to AKAP3 results in inhibition of sperm forward and hyperactivated motility, suggesting that PKA activation and recruitment in the sperm tail by binding to tyrosine-phosphorylated AKAP3 (44) may regulate sperm motility. Moreover, we further showed that tyrosine phosphorylation of AKAP3 occurs physiologically during capacitation following specific activation of SACY by bicarbonate (22) or by inhibition of PI3K activity through LY294002 (26,82). Besides activation of tyrosine kinases, both PI3K inhibition and bicarbonate-stimulated SACY activation also results in a rapid and transient increase in intracellular cAMP levels. This suggests that both signalings may stimulate sperm motility through the selective recruitment and activation of PKA on AKAP3 in the tail and the specific transient and compartmentalized production of cAMP in the fibrous sheath (Fig.6). It may be hypothesized that such a tuned regulatory process is not confined to the sperm tail but also acts in somatic cells. Moreover, stimulation of motility through PI3K inhibition or more generally through AKAP3 induced phosphorylation might allow a more efficient recovery of motile spermatozoa in assisted reproductive techniques (46,54,82). Indeed, the effect of bicarbonate or PI3K inhibition is even more pronounced in both ejaculated and washed spermatozoa obtained from oligoasthenozoospermic rather than normozoospermic subjects (46,54,82).

7. CONCLUSIONS

In conclusion, AKAP scaffolding system is a highly conserved mechanism of initiating focused and compartmentalized signals in the cell. Recent growing literature has revealed a strong involvement of AKAPs not only in somatic cells but in supporting different functions in the developing and mature gamete as well as during fertilization. Interestingly, the male gamete has developed sperm specific AKAP isoforms which strictly regulate sperm functions such as acrosome reaction and motility.

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